Phylogenetic analysis shows that *Bacillus amyloliquefaciens* subsp. *plantarum* is a later heterotypic synonym of *Bacillus methylotrophicus*

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The rhizosphere-isolated bacteria belonging to the *Bacillus amyloliquefaciens* subsp. *plantarum* and *Bacillus methylotrophicus* clades are an important group of strains that are used as plant growth promoters and antagonists of plant pathogens. These properties have made these strains the focus of commercial interest. Here, we present the draft genome sequence of *B. methylotrophicus* KACC 13105T (=CBMB205T). Comparative genomic analysis showed only minor differences between this strain and the genome of the *B. amyloliquefaciens* subsp. *plantarum* type strain, with the genomes sharing approximately 95% of the same genes. The results of morphological, physiological, chemotaxonomic and phylogenetic analyses indicate that the type strains of these two taxa are highly similar. In fact, our results show that the type strain of *B. amyloliquefaciens* subsp. *plantarum* FZB42T (=DSM 23117T=BGSC 10A65) does not cluster with other members of the *B. amyloliquefaciens* taxon. Instead, it clusters well within a clade of strains that are assigned to *B. methylotrophicus*, including the type strain of that species. Therefore, we propose that the subspecies *B. amyloliquefaciens* subsp. *plantarum* should be reclassified as a later heterotypic synonym of *B. methylotrophicus*.

**INTRODUCTION**

*Bacillus methylotrophicus*, isolated from rice rhizosphere soil (Korea), was recently described by Madhaiyan *et al.* (2010). The type strain, KACC 13105T (=CBMB205T), was found to be closely related to members of the *Bacillus subtilis* species complex (see Rooney *et al.*, 2009, for a recent review of this species complex), and showed 16S rRNA gene sequence similarity values ranging from 98.2 to 99.2%. The strain was also shown to be a methylotrophic organism capable of utilizing methanol, triethylamine and ethanol as a sole carbon source.

*Bacillus amyloliquefaciens* was recognized as a distinct species in 1987 and was originally described as a producer of extracellular enzymes (Priest *et al.*, 1987). The species was recently divided into two subspecies, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* and *Bacillus amyloliquefaciens* subsp. *plantarum*, based on complete genome comparisons (Borriss *et al.*, 2011). Strains of the *B. amyloliquefaciens* subsp. *plantarum* clade are plant-associated isolates and typically isolated from the soil or rhizosphere of plants (Borriss *et al.*, 2011; Madhaiyan *et al.*, 2010; Palazzini *et al.*, 2007; Rückert *et al.*, 2011; Schisler *et al.*, 2002). These strains were typically isolated as biological control agents or plant growth promoters.

Over the past decade, considerable interest has been shown in developing *B. methylotrophicus* and *B. amyloliquefaciens* subsp. *plantarum* strains as biological control agents and plant growth promoters (Ongena & Jacques, 2008; Pérez-García *et al.*, 2011; Shan *et al.*, 2013; Sharma & Satyanarayana, 2013; Shi *et al.*, 2014). In recent years, interest in understanding the mode of action of these strains has led to many genomes being published for these strains (Blom *et al.*, 2012; Cai *et al.*, 2014; Dunlap *et al.*, 2013; Geng *et al.*, 2011; Hao *et al.*, 2012; Lefort *et al.*, 2014; Manzoor *et al.*, 2013; Nelson *et al.*, 2014; Niazi *et al.*, 2014a, b; Yang *et al.*, 2011; Zhang *et al.*, 2011, 2014).

**Abbreviations:** ANI, average nucleotide identity; DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession number for the draft genome sequence of *Bacillus methylotrophicus* KACC 13105T is JTKJ00000000. One supplementary figure is available with the online Supplementary Material.
At the time of writing, 28 genome assemblies had been reported for \textit{B. amyloliquefaciens} and two had been reported for \textit{B. methylotrophicus}. In the current study, we report the complete genome sequence of the type strain of \textit{B. methylotrophicus}, strain KACC 13105\textsuperscript{T}. The results of comparisons of this strain with the genomes of closely related taxa show that the \textit{B. amyloliquefaciens} subsp. \textit{plantarum} type strain cannot be distinguished from the type strain of \textit{B. methylotrophicus}. The implications of this finding are discussed herein.

**METHODS**

**Biolog analysis.** \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{Bacillus amyloliquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} were cultured overnight on Biolog universal growth plates and prepared according to manufacturer’s instructions for the GEN III MicroPlate test panel using protocol A. An OmniLog Data Collection instrument (Biolog) was used to collect data in 15 min increments for 22 h.

**Genome sequencing and analyses.** \textit{B. methylotrophicus} strain KACC 13105\textsuperscript{T} was cultured in tryptone-glucose-yeast extract media to early stationary phase (~24 h) and harvested by centrifugation. DNA extraction was performed on the pelleted bacterial biomass using a QIAcube instrument with a QIAmp DNA mini QIAcube kit (QIAGEN). The total genomic DNA extraction was subsequently fragmented to 400 bp using a bioruptor (Diagenode) and size-selected using an E-gel apparatus (Life Technologies). Sequencing adapters were ligated using an Ion Express Plus Fragment Library kit (Life Technologies). Emulsion PCR to incorporate the DNA fragment library to the sequencing beads was performed using the Ion OneTouch instrument with an Ion OneTouch System Template kit (Life Technologies). The library sample was finally sequenced on an Ion Torrent Personal Genome Machine using an Ion 318 chip and the Ion PGM 400 sequencing kit (Life Technologies) following the manufacturer’s suggested protocols. The resulting reads were quality trimmed to the Q20 confidence level. The draft genome was assembled using CLCbio Genomics Workbench 7.1 (QIAGEN) using default parameters.

Genome comparisons and alignments for phylogenetic trees were made using BIGsd software (Jolley & Maiden, 2010). The genomes were downloaded from NCBI and only completed genomes were included using BIGsd software (Jolley & Maiden, 2010). The previously published fatty acid methyl ester data are slightly variable, but the primary fatty acid components are the same. No other notable deviations can be found when comparing the previously published phenotypic, physiological or chemotaxonomic properties of these two taxa (Borriss \textit{et al}., 2011; Madhaiyan \textit{et al}., 2010). As such, we decided to investigate further the extent to which these two taxa can be differentiated using the Biolog GEN III system. Our results are consistent with the aforementioned published studies in that we found no reliable basis for distinguishing these two taxa (Fig. S1, available in the online Supplementary Material). The only notable difference observed in Biolog testing was that \textit{B. amyloliquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} lacked the ability to use maltose. Thus, we decided to conduct a genomic comparison of these two taxa using data from the type strains as well as reference strains.

A draft genome of \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} was assembled from 384160 reads with a mean length of 320 bp. The assembly yielded 105 contigs with a total length of 3876276 bp at 32 \times coverage and 46.4 \% DNA G+C content. The core genome of the combined group of all the \textit{B. methylotrophicus} and \textit{B. amyloliquefaciens} subsp. \textit{plantarum} strains has many distinct features that separate it from the core genome of \textit{B. amyloliquefaciens} subsp. \textit{amyloliquefaciens} strains. These differences have been described extensively in other comparative genomic studies of \textit{B. amyloliquefaciens} strains (Borriss \textit{et al}., 2011; Dunlap \textit{et al}., 2013; He \textit{et al}., 2013; Rückert \textit{et al}., 2011) and remain the same with the inclusion of the \textit{B. methylotrophicus} strains. Specifically, the \textit{B. methylotrophicus}}
Fig. 1. Phylogenetic tree reconstructed from the core genomes of type strains of species from the *Bacillus subtilis* group (1906 genes). Bootstrap values >50%, based on 1500 pseudoreplicates are indicated at branch points. Bar, 0.05 nucleotide substitutions per site.

Fig. 2. Phylogenetic tree reconstructed from the core genomes of strains from *B. siamensis*, *B. amyloliquefaciens* and *B. methylotrophicus* strains (2948 genes). Strain designations are taken from the NCBI accession records. The proposed delineation of the species is provided by the vertical bars. Bootstrap values >50%, based on 1500 pseudoreplicates, are indicated at branch points. Bar, 0.005 nucleotide substitutions per site.
strains possess the non-ribosomal peptide synthase and polyketide synthase operons for macrolactin, difficidin, a representative of the iturin group (such as iturin or bacillomycin) and a representative of the plipastatin group (such as plipastatin or fengycins). In addition, the group lomycin) and a representative of the plipastatin group represent the unique gene clusters for carbohydrate metabolism previously described (He et al., 2013). These conserved features could be adaptations to a plant-associated ecological niche.

To determine if maltose usage was a phenotype specific to \textit{B. methylotrophicus} strains, we conducted a BLAST search of available genomes for the presence of \textit{malL}, the maltase gene in \textit{B. subtilis} required for maltose usage (Schönert et al., 1999). The \textit{malL} gene could be found in \textit{B. methylotrophicus} KACC 13105\textsuperscript{T}, \textit{B. methylotrophicus} JS25R, and several \textit{B. amylo liquefaciens} subsp. \textit{plantarum} strains (B9601-Y2, NAU-B3 and Y2). These results suggest maltose usage is not a distinguishing phenotype.

In order to further characterize the extent to which the above species are differentiated, we conducted phylogenomic analyses of the core genomes of \textit{B. methylotrophicus}, \textit{B. amylo liquefaciens} subsp. \textit{plantarum}, and several other closely related species. The phylogenomic tree based on the core genome (1906 genes) of the strains showed that \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} were very closely related (Fig. 1). The similarity of these closely related genomes was further evaluated using two methods. First, we determined the DDH. The results are reported in Table 1 and show that \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} are highly similar, with a DDH value of 85.1%, which is well above the ~70% threshold for differentiating species. Both \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} had similar values (55.2 and 56.5%, respectively) when compared with \textit{B. amylo liquefaciens} DSM 7\textsuperscript{T}. Both \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} had similar values (56.5 and 56.8%, respectively) when compared with \textit{B. siamensis} KCTC 13613\textsuperscript{T}. The second method used to compare the strains was based on the calculation of the ANI of their core genomes. The results are reported in Table 2 and show that \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} display an ANI of 98.4%. Both \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} displayed similar ANI values (94.3–94.5%) when compared with \textit{B. amylo liquefaciens} DSM 7\textsuperscript{T}. Both \textit{B. methylotrophicus} KACC 13105\textsuperscript{T} and \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T} had similar values (94.6%) when compared with \textit{B. siamensis} KCTC 13613\textsuperscript{T}. The recommended cut-off point of 70% DDH for species delineation corresponds to approximately 95% ANI (Goris et al., 2007).

A second phylogenomic analysis was conducted using the core genome of all published or released \textit{B. siamensis}

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**Table 1. Genome-to-genome distance comparisons of type strains from the \textit{B. subtilis} group**

Regression-based DDH values are indicated.

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<tr>
<td>1. \textit{B. amylo liquefaciens} subsp. \textit{amylo liquefaciens} DSM 7\textsuperscript{T}</td>
<td></td>
<td>56.2</td>
<td>55.2</td>
<td>54.7</td>
<td>20.6</td>
<td>20.4</td>
<td>20.4</td>
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<td>2. \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T}</td>
<td>56.2</td>
<td></td>
<td>85.1</td>
<td>56.8</td>
<td>20.9</td>
<td>20.4</td>
<td>20.5</td>
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<td>3. \textit{B. methylotrophicus} KACC 13105\textsuperscript{T}</td>
<td>55.2</td>
<td>85.1</td>
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<td>20.7</td>
<td>20.4</td>
<td>20.4</td>
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<td>4. \textit{B. siamensis} KCTC 13613\textsuperscript{T}</td>
<td>54.7</td>
<td>56.8</td>
<td>56.5</td>
<td></td>
<td>32.5</td>
<td>31.5</td>
<td>31.5</td>
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<tr>
<td>5. \textit{B. subtilis} KACC 13105T</td>
<td>20.9</td>
<td>20.9</td>
<td>20.6</td>
<td>20.7</td>
<td></td>
<td>87.6</td>
<td>87.5</td>
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<tr>
<td>6. \textit{B. mojavensis} KCTC 3706\textsuperscript{T}</td>
<td>20.4</td>
<td>20.4</td>
<td>20.2</td>
<td>20.4</td>
<td>32.5</td>
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<td>31.5</td>
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<tr>
<td>7. \textit{B. vallismortis} NRRL B-14890\textsuperscript{T}</td>
<td>20.4</td>
<td>20.5</td>
<td>20.2</td>
<td>20.4</td>
<td>42.6</td>
<td>31.5</td>
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**Table 2. ANI comparisons (percentages) of the core genomes between type strains from the \textit{B. subtilis} group**

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<tbody>
<tr>
<td>1. \textit{B. amylo liquefaciens} subsp. \textit{amylo liquefaciens} DSM 7\textsuperscript{T}</td>
<td></td>
<td>94.5</td>
<td>94.3</td>
<td>94.3</td>
<td>82.7</td>
<td>82.4</td>
<td>82.4</td>
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<tr>
<td>2. \textit{B. amylo liquefaciens} subsp. \textit{plantarum} FZB42\textsuperscript{T}</td>
<td>94.5</td>
<td></td>
<td>98.4</td>
<td>94.6</td>
<td>83.0</td>
<td>82.6</td>
<td>82.6</td>
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<tr>
<td>3. \textit{B. methylotrophicus} KACC 13105\textsuperscript{T}</td>
<td>94.4</td>
<td>98.4</td>
<td></td>
<td>94.6</td>
<td>82.4</td>
<td>82.1</td>
<td>81.9</td>
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<tr>
<td>4. \textit{B. siamensis} KCTC 13613\textsuperscript{T}</td>
<td>94.3</td>
<td>94.6</td>
<td>94.6</td>
<td></td>
<td>82.4</td>
<td>82.2</td>
<td>82.4</td>
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<tr>
<td>5. \textit{B. subtilis} KACC 13105\textsuperscript{T}</td>
<td>82.7</td>
<td>83.0</td>
<td>82.7</td>
<td>83.0</td>
<td></td>
<td>88.0</td>
<td>91.4</td>
</tr>
<tr>
<td>6. \textit{B. mojavensis} KCTC 3706\textsuperscript{T}</td>
<td>82.4</td>
<td>82.1</td>
<td>82.1</td>
<td>82.2</td>
<td>87.9</td>
<td></td>
<td>87.5</td>
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<tr>
<td>7. \textit{B. vallismortis} NRRL B-14890\textsuperscript{T}</td>
<td>82.4</td>
<td>82.4</td>
<td>82.1</td>
<td>81.9</td>
<td>91.4</td>
<td>87.6</td>
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Bacillus amyloliquefaciens latter. Thus, we propose that the subspecies Bacillus amylo-
plantarum predates the publication of B. methylotrophicus. The core genome for the group was 2948 genes. The resultant phylogenetic tree is presented in Fig. 2. The analysis shows the existence of three distinct monophyletic clades: one comprised of B. siamensis, the second composed of B. amylo liquefaciens subsp. amylo liquefaciens strains, and the other comprised of B. methylotrophicus and B. amylo-
liquefaciens subsp. plantarum genomes. This latter clade renders B. amylo liquefaciens paraphyletic if the taxonomy is not properly updated on the basis of the results of our analyses. Because the valid publication of B. methylotrophicus predates the publication of B. amylo liquefaciens subsp. plantarum, our results call for the dissolution of the latter. Thus, we propose that the subspecies Bacillus amylo-
liquefaciens subsp. plantarum should be reclassified as a later heterotypic synonym of Bacillus methylotrophicus.

Emended description of Bacillus methylotrophicus Madhaiyan et al. 2010

The description is the same as given by Madhaiyan et al. (2010), except for the following traits. Able to produce macrolactin, difficidin, a representative of the iturin group (such as iturin or bacilomycin) and a representative of the plipastatin group (such as plipastatin or fengycins). Possesses the degradative pathway to metabolize hexuronic acids common in plants.

The type strain is CBMB205^T (=KACC 13105^T=NCCB 100236^T).

This proposal automatically abolishes the subspecies Bacillus amylo liquefaciens subsp. amylo liquefaciens.

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REFERENCES


